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Receptor-Independent Spread of a Highly Neurotropic Murine Coronavirus JHMV Strain from Initially Infected Microglial Cells in Mixed Neural Cultures

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Although neurovirulent mouse hepatitis virus (MHV) strain JHMV multiplies in a variety of brain cells, expression of its receptor carcinoembryonic antigen cell adhesion molecule 1 (CEACAM 1) (MHVR) is restricted only in microglia. The present study was undertaken to clarify the mechanism of an extensive JHMV infection in the brain by using neural cells isolated from mouse brain. In contrast to wild-type (wt) JHMV, a soluble-receptor-resistant mutant (srr7) infects and spreads solely in an MHVR-dependent fashion (F. Taguchi and S. Matsuyama, J. Virol. 76:950–958, 2002). In mixed neural cell cultures, srr7 infected a limited number of cells and infection did not spread, although wt JHMV induced syncytia in most of the cells. srr7-infected cells were positive for GS-lectin, a microglia marker. Fluorescence-activated cell sorter analysis showed that about 80% of the brain cells stained with anti-MHVR antibody (CC1) were also positive for GS-lectin. Pretreatment of those cells with CC1 prevented virus attachment to the cell surface and also blocked virus infection. These results show that microglia express functional MHVR that mediates JHMV infection. As expected, in microglial cell-enriched cultures, both srr7and wt JHMV produced syncytia in a majority of cells. Treatment with CC1 of mixed neural cell cultures and microglia cultures previously infected with wt virus failed to block the spread of infection, indicating that wt infection spreads in an MHVR-independent fashion. Thus, the present study indicates that microglial cells are the major population of the initial target for MHV infection and that the wt spreads from initially infected microglia to a variety of cells in an MHVR-independent fashion.

An initial event in viral infection is the binding of virus to target cells, which is mediated by the binding of a virion surface protein with its specific receptor on the cell membrane. Some molecules classified in the immunoglobulin superfamily are functional receptors for various viruses. CD4 has been identified as a receptor for human immunodeficiency virus (2). In addition, measles virus receptor SLAM (CD150) (34) and carcinoembryonic antigen adhesion molecule 1 (CEACAM1) (mouse hepatitis virus receptor [MHVR]) (4, 36), which serves as a receptor of the murine coronavirus mouse hepatitis virus (MHV), belong to the immunoglobulin superfamily. Although a receptor is an essential molecule in order for a virus to infect, it is not the ultimate determinant for susceptibility of the cell.

MHV, a member of the coronavirus family, is an enveloped virus with single-stranded, positive-sense genomic RNA that is about 30 kilobases long (13). Spike (S) protein, composed of virion projections, is responsible for binding to a receptor and also for the cell entry mechanism of MHV. S protein is a type I glycoprotein of 180 to 200 kDa in molecular mass that is cleaved by host cell-derived protease into two subunits, N-terminal S1 and C-terminal S2 (27). The N-terminal region of the S1, called S1N330, is involved in receptor binding (12, 29), which triggers fusogenic activation accompanied by conformational changes of the membrane-anchored S2 subunit (15). The cell entry mechanism of MHV is thought to be similar to that of human immunodeficiency virus or other enveloped viruses (24).

Four different splice variants of MHVR are known to exist. They have either two or four ectodomains with long or short cytoplasmic tails (1, 4). Two allelic forms have been reported; one is CEACAM1a, which is expressed in most laboratory mouse strains, and the other is CEACAM1b, which is known thus far to be expressed by only the SJL mouse strain (3, 39) but is widely expressed in wild mice (19). A region in the N-terminal domain is responsible for virus binding, which is also involved in the homotypic interaction of this molecule (5, 17, 35).

MHV causes different types of diseases, such as hepatitis, enteritis, and encephalomyelitis. Infection in most tissues is thought to be mediated by MHVR, since the distribution of viral protein in the tissue is closely consistent with the MHVR distribution (9). In contrast, some cells expressing MHVR, e.g., epithelial cells in the kidney or respiratory tracts, have not been reported to be the target of MHV infection (9). Severe encephalomyelitis as a result of extensive infection in various brain cells, such as astrocytes, microglia, ependymal cells, neurons, and oligodendrocytes (14, 20, 21, 28), is observed in infection with wild-type (wt) strain JHMV, although only microglial cells were found to express MHVR (22). The srr7 mutant, derived from wt virus, is benign in terms of neurovirulence (16) compared with wt JHMV, and its infection in the brain is restricted. An outstanding difference in the mode of

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virus spread between wt JHMV and srr7 is that the former spreads in both MHVR-dependent and -independent fashions, whereas srr7 lacks the ability to spread in an MHVR-independent fashion; namely, wt JHMV infection spreads from DBT cells infected via MHVR to MHVR-deficient BHK cells, although srr7 fails to do so (30). The difference in virulence could be due to whether the virus spreads in an MHVR-independent fashion or not, as postulated by Gallagher and Buchmeier (6).

To determine whether the above hypothesis is correct, we have examined in the present study infections with wt virus as well as srr7 in mixed neural cell cultures, which consisted of neurons, astrocytes, microglial cells, and oligodendrocytes. We have confirmed MHVR expression on microglial cells and found that MHVR was a functional receptor. Furthermore, it was revealed that wt virus spreads in neural cell cultures in an MHVR-independent fashion.

MATERIALS AND METHODS

Viruses. A highly neurotropic MHV strain, MHV-JHM cl-2 (designated wt JHMV) (32), and a mutant derived from wt JHMV that is resistant to neutralization by soluble form of MHVR, srr7, were used. srr7 has a single amino acid mutation at position 1114 (Leu to Phe) of the S2 subunit of the S protein relative to wt JHMV (23). These viruses were propagated and titrated using DBT cells maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) as described previously (33).

Cell separation from mouse cerebrum. Specific-pathogen-free ICR mice were purchased from Charles River (Tokyo, Japan). Primary mixed neural cell culture were established from the forebrains of 1- to 3-day-old neonate mice as described previously, with minor modifications (10). Briefly, newborn mice were decapitated, and their cerebral hemispheres were aseptically collected in DMEM. The hemispheres were minced and incubated for 1 h with dispase (Roche, Branchburg, NJ) and collagenase (Invitrogen Corp., Carlsbad, CA) at 37°C with periodic agitation. After centrifugation, the digested tissue was gently triturated with a pipette and washed twice with Hanks' balanced salt solution (HBSS) (Invitrogen Corp.). Myelin, red blood cells, and cell debris were removed from neural cells by using a Percoll (Pharmacia, Uppsala, Sweden) density gradient. After two washings with HBSS, the cells were used for culture or flow cytometry analysis.

Preparation of cultures enriched with microglial cells or astrocytes. For culture of purified microglia, collected cerebral hemispheres were minced, dissociated with trypsin (Invitrogen Corp.), and then maintained with DMEM supplemented with 10% FBS. After 10 days of culture, microglia were detached from an astrocyte monolayer by gently shaking the culture flask. The floating cells were reseeded in a 16-well chamber slide (Nunc, Naperville, IL) in the same medium. For the preparation of astrocyte-enriched cultures, primary neural cell cultures prepared as described above were vigorously shaken to eliminate microglia, oligodendrocytes, and neural progenitor cells growing over the astrocyte monolayer. After shaking, the supernatant was removed and adherent cells were trypsinized and cultured as astrocyte-rich cells.

Effects of CC1 on initial infection of JHMV strains and on subsequent spread of viruses. Cells (1×10^5) isolated as described above were seeded into a well on a polyethyleneimine-coated, 16-well chamber slide and cultured in DMEM supplemented with B27 (Invitrogen Corp.) for 2 days. Neural cells were incubated in 30 μl of DMEM containing serially 10-fold-diluted anti-MHVR monoclonal antibody (MAb) CC1, kindly provided by Kay Holmes (37), at 37°C for 1 h. The same immunoglobulin G1 (IgG1) isotype directed against an irrelevant protein was used as a control. After removal of the culture medium, cells were inoculated with wt JHMV or srr7 at a multiplicity of infection (MOI) of 5 and incubated in the presence of CC1 for 12 h and 24 h after wt JHMV and srr7 infection, respectively. Cells were then fixed with 4% paraformaldehyde, and viral antigens in cells were visualized by immunohistochemistry using anti-MHV MAb (11) and anti-mouse IgG labeled with horseradish peroxidase. Antigen-positive cells were counted under microscopy. The blockade of viral infection by CC1 was calculated as the percentage of viral antigen-positive cells in the presence of each dilution of CC1, compared with cell numbers obtained in the absence of CC1.

To examine whether CC1 inhibits virus spread from initially infected cells, mixed neural cell cultures, as well as microglial cell-enriched cultures, prepared as described above, were infected with either wt virus or srr7 at an MOI of 0.5 and incubated at 37°C for 1 h. Cells were then washed with HBSS and cultured in the presence or absence of CC1 (1 μg/ml) for 24 h. Viral antigens in cells were observed as described above.

Immunocytochemistry. Primary antibodies used to identify each cell type were as follows: anti-glial fibrillary acidic protein (DAKO Chemical, Denmark) polyclonal antibody for astrocytes, O4 MAb (Chemicon, CA) for oligodendrocytes, and MAP-2 (Chemicon Int. Inc., Temecula, CA) for neurons. Binding of the *Griffonia simplicifolia* lectin (GS-lectin) (Vector Laboratories) was used for microglial cell identification. To detect MHV-specific antigen in cells, mouse anti-MHV MAbs were used. Cells grown on a polyethyleneimine-coated Lab-Tek 16-chamber slide were fixed in 4% paraformaldehyde for 10 min and permeabilized by exposure to 1% Triton X-100 for 60 min. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide in phosphate-buffered saline, pH. 7.2. Before staining with mouse MAb, nonspecific binding was blocked using Mouse on Mouse (Vector Laboratories), and antibody binding was detected with avidin-biotin complex (Vector Laboratories), using amino-ethyl-carbazol (Vector Laboratories) as the chromogen. Finally, cells were counterstained with hematoxylin prior to mounting. In order to identify the MHV-infected cells, immunofluorescence dual staining with viral antigen and a cell-specific marker was employed.

Flow cytometry. Flow cytometry (fluorescence-activated cell sorting [FACS]) was employed for detecting the expression of CEACAM1 and viral attachment to the cell surface and the blockade of the viral attachment by MAb CC1. Nonspecific binding on cerebral cells separated as described above was minimized by blocking with purified rat anti-mouse FcIII/IIR MAb (2.4G2; BD PharMingen, San Diego, CA) and PBS containing 1% FBS for 10 min prior to staining. To detect the viral receptor CEACAM1on the cell surface, cells were incubated in CC1 for 30 min at 4°C, or designated IgG1 isotype-matched controls (BD PharMingen), followed by biotinylated anti-mouse IgG1 and avidin-conjugated phycoerythrin (PE). To detect cell-bound wt JHMV or srr7, the cells were infected at an MOI of 5 and incubated for 1 h at 4°C. Cell-bound viruses were detected with anti-MHV MAb and anti-mouse IgG labeled with Alexafluor 488 (Molecular Probes, Inc., Eugene, OR). Virus-attached cells were dual stained with Alexafluor 488-labeled MAb and an antibody for a cell surface maker. To evaluate the competition by CC1 and virus for the receptor, the cells were pretreated with CC1 for 30 min at 4°C. After three washes, they were incubated with wt JHMV or srr7 for 1 h at 4°C, and this was followed by the addition of a mixture of biotinylated anti-mouse IgG1 and Alexafluor 488-labeled MAb against MHV. In addition, after three washes, avidin-PE was added. All stained cells were detected on a FACScan (Becton, Dickinson and Co., NJ) and analyzed with CellQuest software.

Confocal microscopy. Single-plane confocal microscope images were collected using a microscope with an attached krypton/argon mixed-laser unit (Yokogawa Electric Corp.). The gas laser produced excitation wavelengths of 488 nm and 568 nm for Alexafluor 488 and Texas Red, respectively. Individual fluorophore images were merged and pseudocolored using Adobe Photoshop (version 5.5).

RESULTS

Infection of wt JHMV or srr7 in cultured neural cells and blockade of viral infection by MAb CC1. We prepared mixed neural cell cultures from neonatal mouse brain and cultured them for 2 days before virus challenge. To reveal the cell population, we stained the cultivated cells with cell type-specific antibodies or reagents as described in Materials and Methods. Staining revealed that about 40% of the population consisted of neurons; 20%, astrocytes; 20%, microglia; and 1 to 2%, oligodendrocytes. Neuronal progenitor cells were roughly 25% of the total under these culture conditions. We infected the cultivated cells with wt JHMV or srr7, and viral antigens expressed in these cells were monitored by immunocytochemistry using MHV-specific MAbs. As shown in Fig. 1A, viral protein was sporadically detected at 8 h after infection with wt JHMV. The infected cells eventually formed syncytial clusters composed of several cells. The infection rapidly spread with intercellular fusion formation regardless of cell type, and a number of syncytia were observed at 12 h. Twenty-four hours

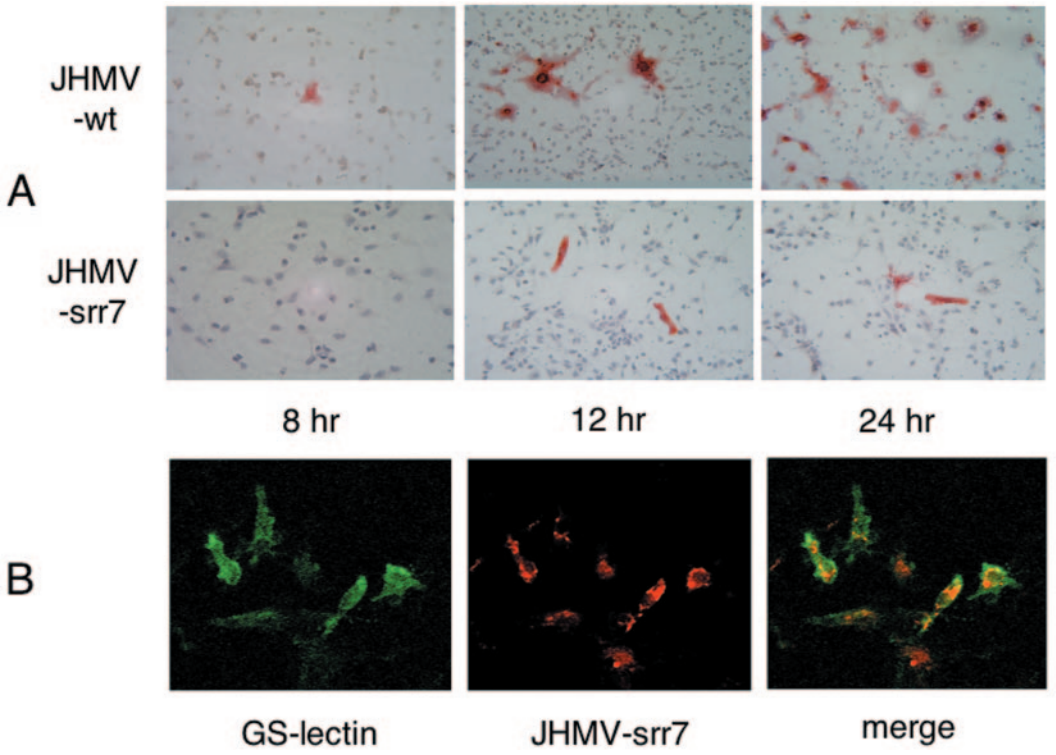

FIG. 1. (A) Infection of mixed neural cell cultures with either wt JHMV or srr7. Mixed neural cell cultures were infected with either wt JHMV or srr7 and fixed with 4% paraformaldehyde at 8 h, 12 h, or 24 h after infection. Viral antigens in cells were detected by staining with anti-MHV MAbs and anti-mouse IgG. (B) Immunofluorescent double staining of srr7-infected cells with anti-MHV MAbs and microglial cell/macrophage-specific GS-lectin. Mixed neural cell cultures were infected with srr7 and incubated at 37°C for 12 h. Viral antigens, as well as microglial cells, were stained with anti-MHV MAbs (Alexafluor 488, green) and GS-lectin (Texas Red, red), respectively, and the images were merged.

after infection, the majority of cells were infected and large syncytia were formed. Unlike those in wt JHMV-infected cells, the viral proteins in srr7-infected cells were not detected as early as 8 h postinoculation. At 12 h viral antigens were observed in only a small proportion of club-like cells, which were morphologically similar to microglial cells. However, srr7 did not induce any intercellular fusion even 24 h after infection. To address whether the cells infected with srr7 observed at 12 h after infection are microglial cells or not, we doubly stained those cell cultures with anti-MHV MAb and GS-lectin, a microglial cell/macrophage marker. Cells with MHV antigen were also stained with GS-lectin (Fig. 1B), which suggested that microglial cells are initially infected by srr7.

We further examined whether the infections caused by these viruses were mediated by MHVR. At first, cell cultures were pretreated with serially diluted anti-MHVR MAb CC1 and challenged with wt JHMV and srr7. Infections in these cells were then monitored by the presence of viral antigens. As shown in Fig. 2, infection with either virus was blocked by pretreatment of CC1 in a concentration-dependent fashion. CC1 at a concentration of 1 to 10 μg/ml (dilution of 10^3 to 10^2) almost entirely prevented infection by either wt JHMV or srr7. It was thus concluded from these observations that the initial infections with wt or srr7 JHMV in neural cells are mediated by MHVR, making the process similar to that with MHV infection in hepatocytes or lymphoid tissue (9).

Identification of cell types expressing MHVR and those to which the viruses attach. The data above indicated that wt JHMV infected various neural cells, while srr7 infected at least microglial cells. Since the wt infection could result from

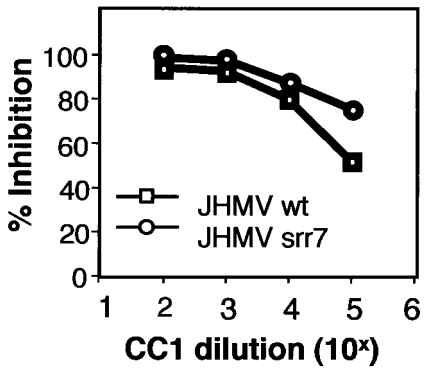

FIG. 2. Blockade of wt and srr7 infection in mixed neural cell cultures by anti-MHVR MAb CC1. Mixed neural cell cultures were treated with 10-fold-stepwise-diluted CC1 at 37°C for 1 h and then infected with either wt virus or srr7. After infection, cells were cultured in the presence of CC1. Viral antigen was visualized by staining with anti-MHV MAbs at 12 and 24 h after wt and srr7 infection, respectively, and antigen-positive cells were counted. The blockade of viral infection by CC1 was calculated as the percentage of viral antigen-positive cells in the presence of CC1 compared with cell numbers obtained in the absence of CC1.

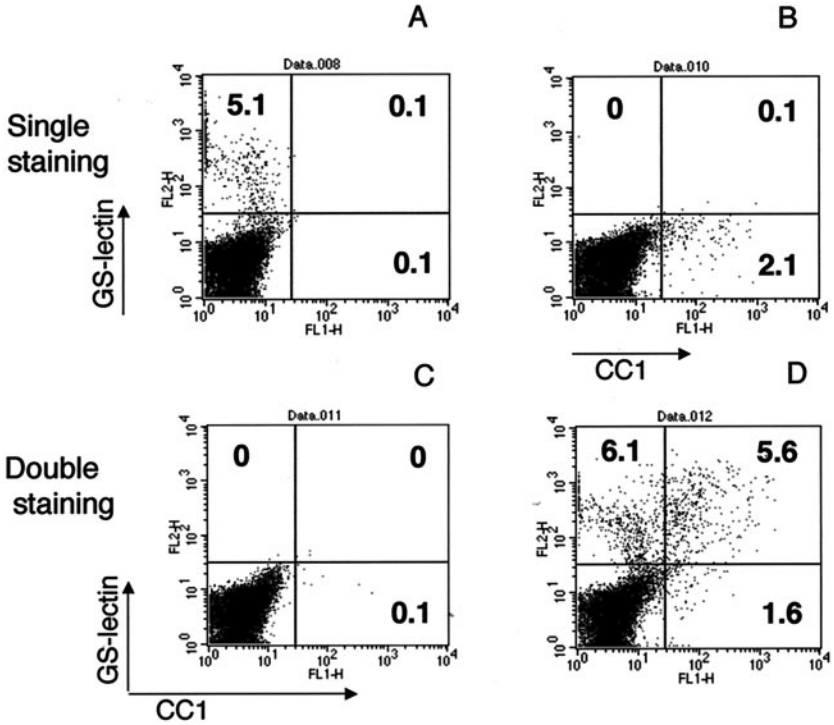

FIG. 3. Examination of MHVR-bearing cells by flow cytometric analysis. Neural cells separated from cerebrums of 1- to 3-day-old mice were labeled with either GS-lectin (PE) (A) or anti-MHVR MAb CC1 (Alexafluor 488) (B). Cells were also analyzed for coexpression of GS-lectin and MHVR (D) by double staining as described in Materials and Methods. (C) Background control.

MHVR-independent infection, we have focused on the microglial cells that were infected by srr7. Thus, microglial cells were first evaluated by FACS as MHVR-bearing cells. Cells isolated from the cerebrums of neonatal mice were directly examined for the presence of MHVR by using CC1. Simultaneously, those cells were also examined by using GS-lectin binding. MHVR-expressed and GS-lectin-binding cells were revealed to consist of 2.1% and 5.1% of the total cells, respectively (Fig. 3). Double staining with CC1 and GS-lectin revealed that approximately 78% of the MHVR-bearing cells were GS-lectin-positive microglial cells. These results also demonstrated that half of the microglial cells are MHVR positive, indicating that the major population expressing MHVR consists of microglial cells.

We further examined, using FACS, the cell types that permit attachment of JHMV as well as those that express GS-lectin. Neural cells prepared as described above were mixed with wt JHMV, and cells bound by viruses were monitored by anti-spike MAbs. It was demonstrated that the percentages of cells allowing the attachment of JHMV and those that were GS-lectin positive were 1.2% and 3.8%, respectively (Fig. 4A and B). It was also shown that double-positive cells consisted of 1%, covering more than 83% of the cells bound by virus, suggesting that most of the cells bound with JHMV are microglial cells. We then examined by FACS whether or not pretreatment of these cells with CC1 prevents JHMV attachment on the cell surface. Cells were first treated or not treated with CC1 at 4°C and then infected with wt JHMV. The binding of CC1 and that of JHMV were determined by using antibodies against CC1 and JHMV, respectively. Figure 4C, D, and E show that the percentage of cells allowing JHMV attachment decreased from 1.3% without treatment with CC1 to almost 0% if these cells were pretreated with CC1, indicating that wt JHMV attaches to neural cells via MHVR but not through other molecules on the cell surface.

Taken together, these findings suggest that both wt and srr7 initially infect microglial cells via MHVR expressed on their surface, and there is very little chance that JHMV infection is mediated by molecules other than MHVR. It is also suggested that MHVR expressed on cells other than microglial cells (consisting of 22% of cells expressing CEACAM1) would play a role in the initial infection with JHMV.

Virus infection in cultures enriched with microglial cells. The results described above suggested that MHVR-positive cells, mostly microglial cells, are initial target cells for wt and srr7 in mixed neural cell cultures. It is also postulated that wt JHMV spreads in an MHVR-independent manner but that srr7 fails to do so in mixed neural cell cultures. If this is the case, both of these viruses are thought to spread efficiently in cell cultures enriched with microglial cells, half of which are shown to be MHVR positive. A culture established by shaking the mixed neural cells usually contains more than 95% GS-lectin positive microglial cells. We infected this culture with either wt JHMV or srr7 and monitored viral spread by the appearance of syncytial cells as well as viral antigens in cells. As shown in Fig. 5, cultures prepared as described above consisted predominantly of microglial cells as revealed by GS-lectin-positive binding. In this culture, both wt and srr7 produced similarly large numbers of syncytia, including approximately 50% of the cultured cells, and those cells were also GS-lectin

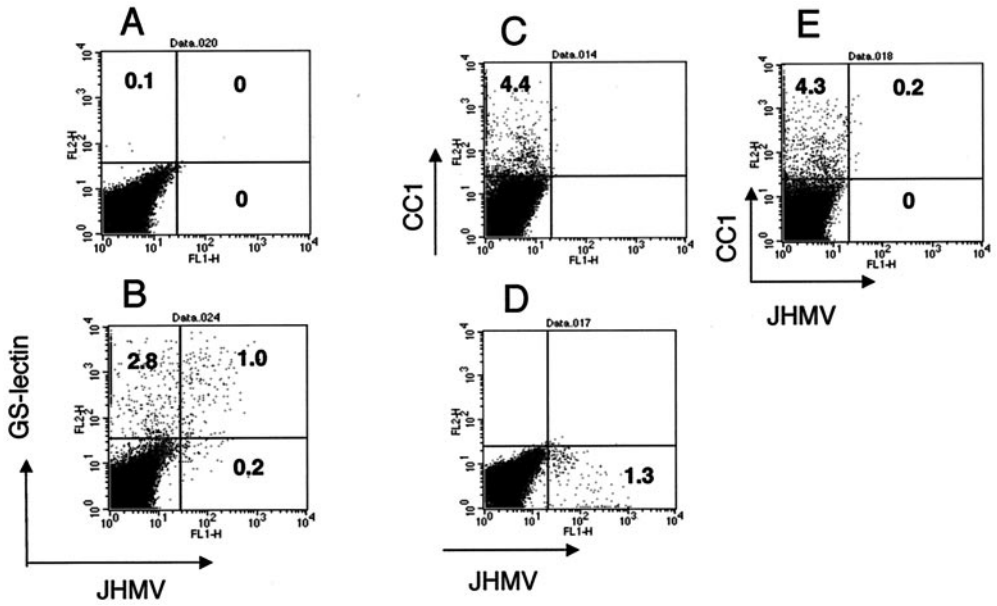

FIG. 4. (A and B) Detection of MHVR and JHMV binding to cells by flow cytometric analysis. Cells isolated from neonate mouse brain were mixed with wt JHMV and stained with anti-MHV MAb–Alexafluor 488. Those cells were also examined for binding by GS-lectin PE and analyzed by flow cytometry (B). (A) Background control. (C to E) Inhibition of wt JHMV attachment on neural cells by CC1. Cells isolated from neonatal mice were first treated with CC1 at 4°C for 30 min and then infected with wt JHMV. JHMV attached to cells was detected with anti-MHV MAb, biotinylated anti-mouse IgG1, and avidin-PE (E). As a control, cells single stained with CC1 (C) or those with attached virions (D) were also analyzed by flow cytometry.

positive. These findings indicated that both viruses spread and induced syncytia efficiently in microglial cultures, in a manner similar to that of infection in MHVR-positive DBT cells.

Virus infection in astrocyte-enriched cultures. Several reports have described that astrocyte-enriched cultures support JHMV infection (21), although microglial cell contamination in this culture was not thoroughly excluded. We infected astrocyte-enriched cultures, prepared as described in Materials and Methods, with wt and srr7. As shown in Fig. 6A, wt infection induced syncytium formation. Although srr7 failed to induce syncytia, viral infection was evidenced by detection of the antigen in a few cells infected with srr7 (Fig. 6A). These ob-

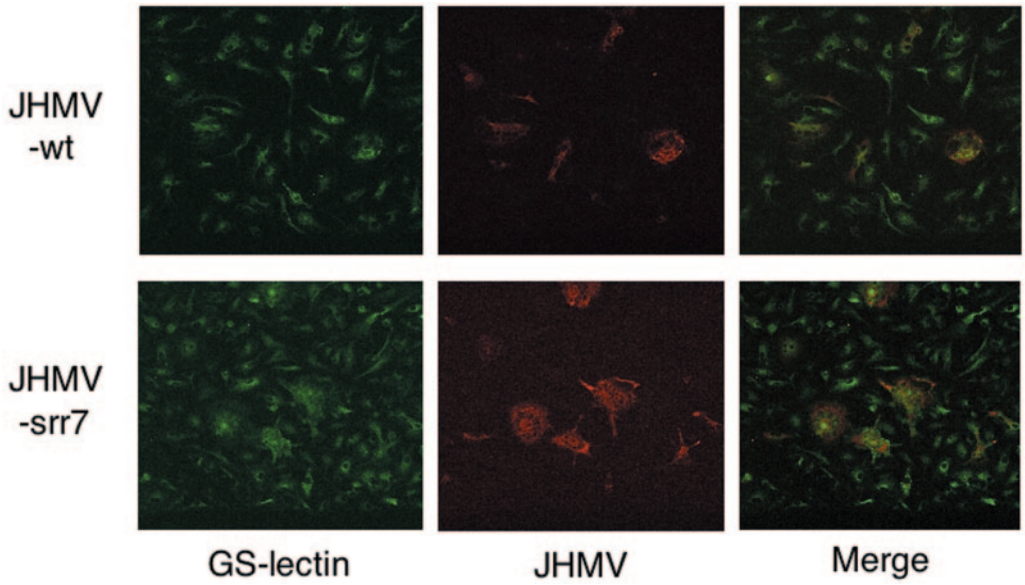

FIG. 5. Infection of microglial cell-enriched cultures by wt JHMV or srr7. Microglial cell-enriched cultures were prepared as described in Materials and Methods. Those cells were infected with either wt JHMV or srr7, and viral antigens were examined with anti-MHV MAb (Texas Red) as well as for the presence of GS-lectin (Alexafluor 488) on the cultured cells at 12 h after infection. The images of GS-lectin-positive and MHV-positive cells were merged.

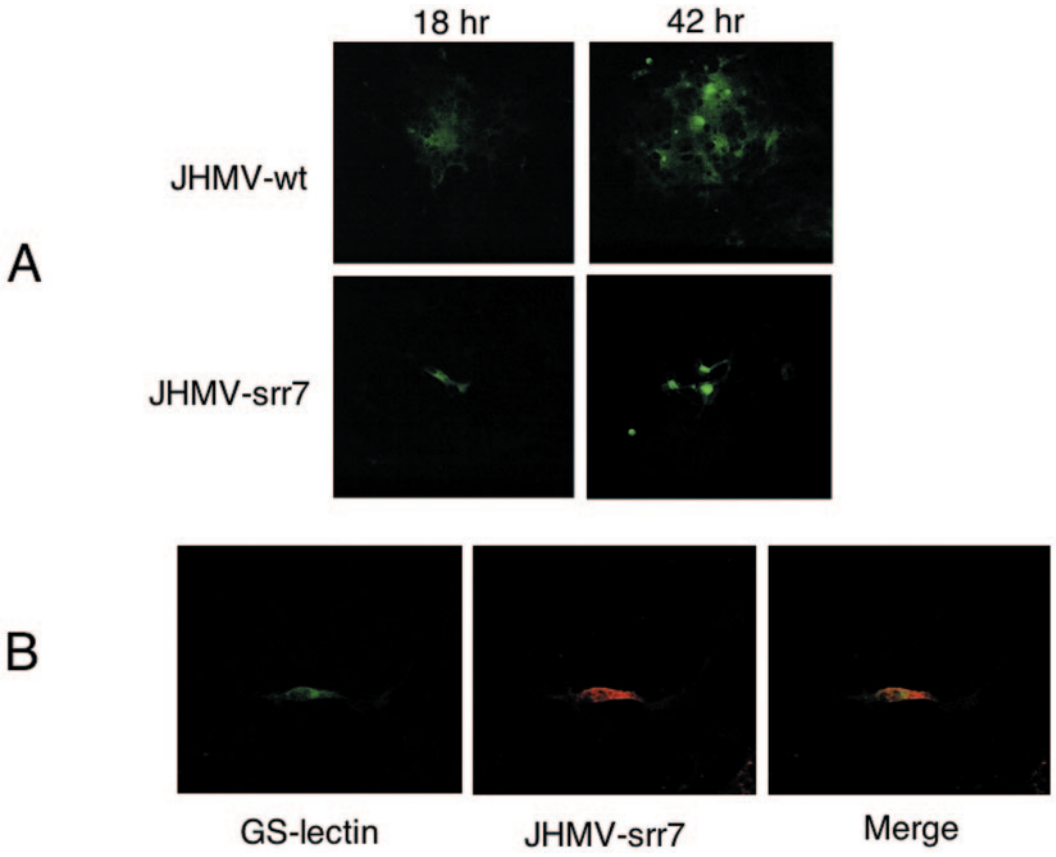

FIG. 6. (A) Infection of astrocyte-enriched cultures by wt and srr7. Astrocyte cultures were prepared as described in Materials and Methods. Cells were infected with either wt JHMV or srr7, and viral antigens were monitored with anti-MHV MAb (Alexafluor 488) at 12 h after infection. (B) Infection of srr7 in astrocyte-rich cultures. srr7-infected cells in astrocyte-rich cultures were also examined for GS-lectin positivity (using Texas Red) as well as with anti-MHV MAb. These two images were merged.

servations were very similar to those recorded for infection in mixed neural cell cultures. We next tried to test whether srr7-infected cells are microglial cells or not. As shown in Fig. 6B, srr7 antigen-positive cells were revealed to be GS-lectin positive, showing that srr7 infected solely microglial cells mingled within the astrocyte culture. This strongly suggests that initial infection in astrocyte culture takes place in microglial cells as well. Wt infection could spread to astrocytes in an MHVR-independent fashion, while srr7 infection was restricted among microglia.

Effects of CC1 treatment on virus spread from initially infected cells. The data presented above suggest that wt virus spreads from initially infected microglial cells to MHVR-negative cells existing in mixed neural cell cultures, while srr7 infection is restricted to MHVR-positive microglial cells. To see whether this is the case or not, we examined whether CC1 could inhibit the spread of wt infection from the initially infected cells. Mixed neural cell cultures infected with wt virus 1 h previously were cultured with medium in the presence or absence of CC1. Cells were then immunochemically stained using anti-MHV MAb to see the spread of virus infection. As shown in Fig. 7A, there was no substantial difference in the proportion of MHV antigen-positive syncytia between cells cultured in the presence and absence of CC1, indicating that wt virus spread from initially infected cells was not prevented by CC1. We also did a similar experiment using either wt- or srr7-infected microglial cell cultures, and about 50% of those expressed MHVR. As shown in Fig. 7B, CC1 prevented the spread of srr7, while it failed to prevent the wt virus spread in a way observed in srr7 infection, although CC1 treatment reduced wt syncytium formation to a certain extent. This reduction could be due to the prevention of an MHVR-dependent infection in microglial cells. These results clearly showed that wt virus spread from initially infected cells to a variety of cells in an MHVR-independent fashion, while srr7 infection was solely MHVR dependent.

DISCUSSION

The neurotropic MHV strain JHMV induces encephalomyelitis by infecting a wide spectrum of brain cells (14, 20, 25, 28), although except for microglia, MHVR has not been reported to exist on these neural cells (22). Extensive replication of highly neurotropic JHMV in various cells in the mouse brain could be due to the existence of another functional MHV receptor different from MHVR. However, in vivo and in vitro experiments have shown that infection of glial progenitors, oligodendrocytes and astrocytes, was blocked by pretreatment with anti-MHVR antibody MAb CC1 or Ab-655 (9), strongly

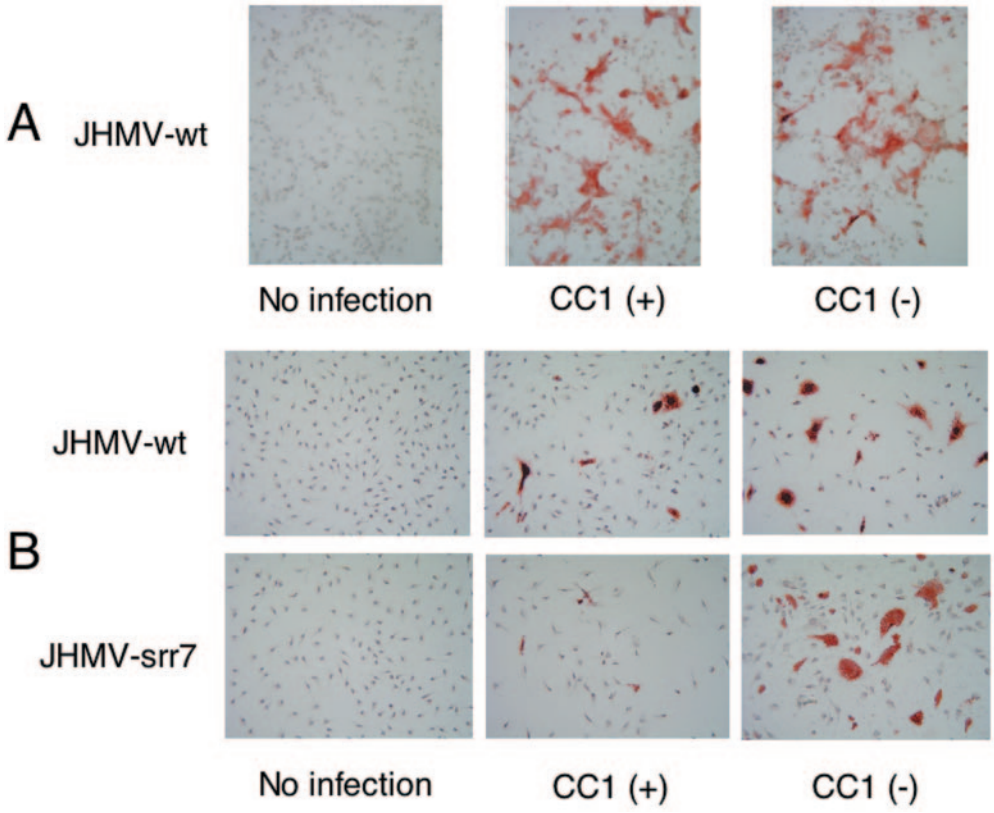

FIG. 7. (A) Effect of CC1 on wt virus spread from initially infected cells. Mixed neural cell cultures were infected with wt virus. After 1 h of adsorption, cells were washed with HBSS and cultured in the presence or absence of CC1. Cells were fixed at 24 h after infection and stained with anti-MHV MAbs. (B) Effect of CC1 on the spread of wt virus and srr7 from initially infected cells in microglial cell-enriched cultures, which were infected with either the wt or srr7 and incubated at 37°C for 1 h. After washing, cells were cultured with medium in the presence or absence of CC1. Cells were stained with anti-MHV MAbs at 24 h after infection.

suggesting that MHVR is essential for the initiation of MHV infection in the brain.

In the present study, we have confirmed the observation of Ramarkrishna et al. (22) that MHVR is expressed on microglial cells, and we have further revealed that MHVR on microglia is a functional receptor. These observations strongly suggest that microglial cells are an initial target of MHV in mixed neural cell cultures and the brain as well. We furthermore showed that wt virus spread from initially infected microglial cells to other types of neural cells without MHVR expression in an MHVR-independent fashion, while srr7 virus lacked this activity. An MHVR-independent infection could contribute to the high neurovirulence of wt virus for mice by infecting a wide spectrum of neural cells in the brain, as was previously suggested by Gallagher and Buchmeier (6).

In double-staining experiments, FACS analysis showed that most, if not all, MHVR-bearing cells were GS-lectin-positive microglial cells. It was further shown that the virus attached to the cells expressing MHVR, since attachment of JHMV on cells was blocked by anti-MHVR antibody CC1. MHVR was also crucial for virus replication, since infection was prevented by pretreatment of cells with CC1. These findings strongly suggest that microglial cells are the major initial targets of JHMV. It should also be noted that 20% of the MHVR-positive cells are GS-lectin negative. These unidentified cells could also serve as initial target cells. This population possibly consists of neurons, astrocytes, oligodendrocytes, neural progenitor cells, ependymal cells, or lectin-negative microglial cells. However, in the mixed cell cultures, srr7 did not infect astrocytes or oligodendrocytes, suggesting that it is unlikely that those cells serve as initial targets of JHMV.

The differences in infection in mixed neural cell cultures observed between the wt and srr7 viruses are in the stage of spread from initially infected cells to MHVR-negative cells, since the initial infection with those viruses takes place in a similar fashion, as blocked by CC1 treatment. If some molecule is involved in the spread, then the wt virus would have access to a variety of cells via such a molecule, but srr7 does not. Although we cannot thoroughly rule out such a possibility, this variation could be explained by their different modes of infection, namely, MHVR-independent infection by wt virus versus MHVR-dependent infection by srr7. The minor effect of CC1 treatment on wt virus spread from initially infected cells to the whole culture clearly indicates the MHVR-independent spread of wt virus, while the inhibitory effect of CC1 on srr7 infection in microglial cell cultures suggests an MHVR-dependent spread of srr7. These differences are similar to those observed in cultured cell lines (7, 18, 30, 31).

Microglia have various markers, depending on their different stages, and they retain functional changes similar to those

seen in other tissue macrophages. Of the various markers, the GS-lectin marker was used in this experiment, because it is expressed on microglial cells throughout all of their stages (26, 38). However, it is also one of the markers for endothelial cells (26), and those cells, in the brain, have been already reported to express MHVR and to be attached by the virus as determined by a direct binding assay on thin sections (8). The cultures prepared in our experiment did not include endothelial cells, as examined by using factor VIII-positive cells (data not shown), a marker of endothelial cells. Thus, the GS-lectin-positive cells used in our study were considered microglial cells.

This study shows that either strain of the virus attached to only 1 to 2% of the whole cells separated from the mouse cerebrum. This number represents less than half of the MHVR-positive cells. Godfraind et al. (9) have reported the existence of MHVR-bearing cells but not susceptible cells such as those of the thyroid gland or epithelial cells of the respiratory tract. It is not evident at present what causes this observed difference between susceptible and unsusceptible MHVR-bearing cells, but it could be possible that some other, as-yet-unidentified, host factor(s) could contribute to the sensitivity to MHV infection.

This study showed that wt virus spreads to various types of cells in culture, and conceivably in the mouse brain, by an MHVR-independent mechanism of infection. Studies are in progress to test whether or not MHVR-independent infection contributes to the high neuropathogenicity of wt JHMV in mice.

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